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Nucleolar targeting: the hub of the matter

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The nucleolus is a dynamic structure that has roles in various processes, from ribosome biogenesis to regulation of the cell cycle and the cellular stress response. Such functions are frequently mediated by the sequestration or release of nucleolar proteins. Our understanding of protein targeting to the nucleolus is much less complete than our knowledge of membrane-spanning translocation systems—such as those involved in nuclear targeting—and the experimental evidence reveals that few parallels exist with these better-characterized systems. Here, we discuss the current understanding of nucleolar targeting, explore the types of sequence that control the localization of a protein to the nucleolus, and speculate that certain subsets of nucleolar proteins might act as hub proteins that are able to bind to multiple protein targets. In parallel to other subnuclear structures, such as PML bodies, the proteins that are involved in the formation and maintenance of the nucleolus are inexorably linked to nucleolar trafficking.

Keywords: hub protein; nucleolar localization; nucleolin; nucleolus; nucleophosmin

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See Glossary for abbreviations used in this article.

Introduction

The nucleus is a highly ordered structure that contains non-membrane-bound subcompartments—including PML bodies, splicing speckles, Cajal bodies and the nucleolus—that have specific functions. The nucleolus is the largest subnuclear structure (Fig 1) and is easily visible under the light microscope owing to its high refractive index. It is centred on rDNA repeats within the chromosomes and is traditionally associated with ribosome biogenesis. In mammalian cells, the number and activity of nucleoli vary during the cell cycle according to differing metabolic conditions and cell types.

The mammalian nucleolus can be morphologically divided into several discrete regions—the fibrillar centre (FC), the dense fibrillar centre (DFC) and the granular component (GC)—that have roles in the various steps of rRNA synthesis. The FC contains the transcription factor UBF and is rich in RNA polymerase I. The DFCs are associated with, and surround, the FCs and contain fibrillarin, an RNA methyltransferase and nucleolin—a protein that has multiple roles in nucleolar and cellular biology (Mongelard & Bouvet, 2007). Surrounding both the FC and the DFC is the GC, which is the site of the partial maturation and assembly of pre-ribosomes, accumulates NPM, and is enriched with ribosomal proteins and assembly factors. The GC might also contain regions that comprise protein complexes that are devoid of RNA (Politz *et al*, 2005).

Our understanding of nucleolar function changed markedly with the formulation of the plurifunctional nucleolus hypothesis (Pederson, 1998), which has now been underpinned by proteomic analysis (Boisvert *et al*, 2007), and proposes that the nucleolus has multiple functions in health and disease (Matthews & Olson, 2006; Stark & Taliansky, 2009). For example, there is extensive evidence that the nucleolus is involved in the response to cellular stress (Mayer & Grummt, 2005; Rubbi & Milner, 2003), and in the regulation of the cell cycle and cell growth (Martindill & Riley, 2008). The nucleolus can also be a target for virus infection (Hiscox, 2007) and perturbations to the nucleolus have been observed in a wide range of cellular diseases, from auto-immunity to cancer (Montanaro *et al*, 2008).

The regulation of many nucleolar roles is mediated by the sequestration or release of specific proteins, thereby providing insight into one of the mechanisms by which the nucleolus operates. This is illustrated by the sequestration of NF-κB (RelA) to the nucleolus in colorectal cancer cell lines in the presence of non-steroidal anti-inflammatory drugs such as aspirin, which induces apoptosis (Thoms *et al*, 2007). Another implication of continuous protein sequestration and release is that the nucleolar proteome is continuously changing in response to metabolic conditions and driving metabolic change (Lam *et al*, 2007). For example, the ribosomal—and part nucleolar—protein L23 is retained in the nucleolus by NPM, thereby enhancing MIZ1-mediated cell-cycle arrest (Wanzel *et al*, 2008). NPM also sequesters the ribosomal protein S9 in nucleoli and directly affects ribosome biogenesis (Lindstrom & Zhang, 2008). The localization and stability of nucleolin—one of the most abundant nucleolar proteins—can also change in response to differing metabolic conditions such as osmotic changes (Yang *et al*, 2008) and developmental stages (Bicknell *et al*, 2005). Similarly, alterations in cellular pH—for example,

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Glossary

ARF	alternative reading frame product of the *CDKN2A* locus
dsRNA	double-stranded RNA
EGFP	enhanced green-fluorescent protein
FGF2	fibroblast growth factor 2
HIF	hypoxia-inducible factor
HIV-1	human immunodeficiency virus type 1
HSV-1	herpes simplex virus type 1
HVS	herpesvirus saimiri
MIZ1	Myc-associated zinc-finger protein
NF-κB	nuclear factor-κB
NOM1	nucleolar protein with MIF4G domain 1
NPM	nucleophosmin, also known as B23.1 (there is also an alternative splice variant called B23.2)
NRF	nuclear factor-κB-repressing factor
ORF	open reading frame
PML	promyelocytic leukaemia protein
PP1	protein phosphatase 1 (a serine/threonine phosphatase)
PRRSV	porcine reproductive and respiratory syndrome virus
rDNA	ribosomal DNA
RelA	nuclear factor-κB p65/Rel A
rRNA	ribosomal RNA
SUMO	small ubiquitin-like modifier
UBF	upstream binding factor
UL24	unique long 24
US11	unique short 11
VHL	von Hippel–Lindau tumour-suppressor protein

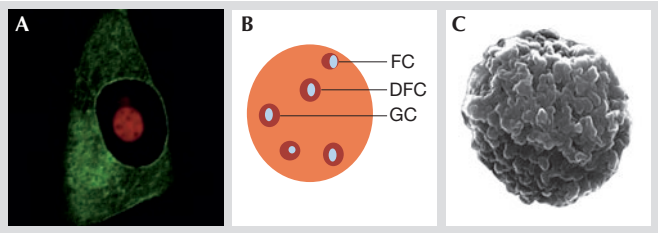

Fig 1 | Structure of the nucleolus. (**A**) Live-cell laser-scanning confocal microscope image showing the localization of a fluorescently tagged nucleolar marker protein (red) with a fluorescently tagged cytoplasmic marker protein (green). The nucleolus constitutes a significant proportion of the nucleus and contains defined features. (**B**) Diagrammatic representation of the mammalian nucleolus showing the positions of the FC, DFC and GC. (**C**) Scanning electron micrograph of a nucleolus purified from HeLa cells. The surface corresponds to a shell of highly condensed heterochromatin that surrounds the nucleolus *in vivo* (image courtesy of Angus Lamond, University of Dundee, UK). DFC, dense fibrillar component; FC, fibrillar centre; GC, granular component.

owing to hypoxia or acidosis—have been shown to cause the nucleolar sequestration of the VHL protein (Mekhail *et al*, 2004), one function of which is to degrade HIF, which activates genes involved in tumour vascularization and oxygen homeostasis. Therefore, the sequestration of VHL to the nucleolus during hypoxia or acidosis—which is mediated by a H^+-responsive nucleolar localization signal (NoLS)—allows the activation of HIF until normal cellular conditions are reached, which results in the release of VHL from the nucleolus and the subsequent inhibition of HIF function (Mekhail *et al*, 2004).

The continuous exchange of nucleolar proteins with the nucleoplasm and cytoplasm in response to fluctuating cellular conditions indicates that nucleolar proteins might have specific signals or pathways that determine nucleolar sequestration or release.

Hub proteins in the nucleolus?

The nucleolus consists of complex protein–protein and protein–nucleic acid interactions, and, although it is imaged as a steady-state structure, high-throughput proteomic analysis of purified nucleoli coupled with live-cell imaging of fluorescently labelled nucleolar proteins has revealed a dynamic nuclear compartment, the components of which undergo continuous exchange with the nucleoplasm (Andersen *et al*, 2005; Hernandez-Verdun, 2006). The current human nucleolar proteome database—which has an estimated 80% coverage—consists of more than 4,500 proteins (Ahmad *et al*, 2009). Many nucleolar proteins interact with one another, and with other nuclear and cytoplasmic proteins depending on trafficking and the cell-cycle stage (Fig 2), and perturbations to certain proteins in the nucleolus—either enrichment or ablation—have a knock-on effect on partner proteins and, hence, function. The nucleolus is assembled in mitosis, when nucleolin, NPM, fibrillarin and other factors form nucleolus-derived foci (Hernandez-Verdun *et al*, 2002). Therefore, members of a subset of the nucleolar proteome act as building blocks of the nucleolus around rDNA repeats, and it is formed in an incremental manner (Dundr *et al*, 2000). For assembly to occur in this way, these blocks must bind to multiple partner molecules, thereby probably functioning as so-called hub proteins.

A common characteristic of hub proteins is the ability to bind to 10 or more distinct proteins (Krasowski *et al*, 2008). Hub proteins have been extensively studied in *Saccharomyces cerevisiae*, in which two types have been identified (Ekman *et al*, 2006). Dynamic hub proteins are proposed to bind to different partner molecules at different times or subcellular locations, whereas static hubs interact with most partners simultaneously. Traditionally, hub proteins have been thought to be important components of protein–protein interaction networks; however, the possibility that their capacity to interact with large numbers of target proteins could also have a structural role has so far been neglected. The nucleolus and other nuclear structures without membranes are dynamic, with their constituent proteins in constant flux. Therefore, if a hub protein is present in one compartment in sufficient quantity, we speculate that sequential transient interactions between the targeted protein and multiple hub proteins could be sufficient to induce a period of residence within the compartment. Hub proteins are known to facilitate interactions with a wide range of partner proteins (Kim *et al*, 2008), while retaining the ability to distinguish between suitable partners. In this case, localization works through the recognition of structural or sequence-based motifs within a protein, which make possible its identification as a protein that must be retained within a specific subcellular localization.

Hub proteins might be responsible for much of the nucleolar localization that occurs in the absence of protein–RNA interactions. Each hub protein could have different recognition requirements and/or multiple recognition sites, thereby explaining the existence of different NoLSs. This hypothesis is supported by

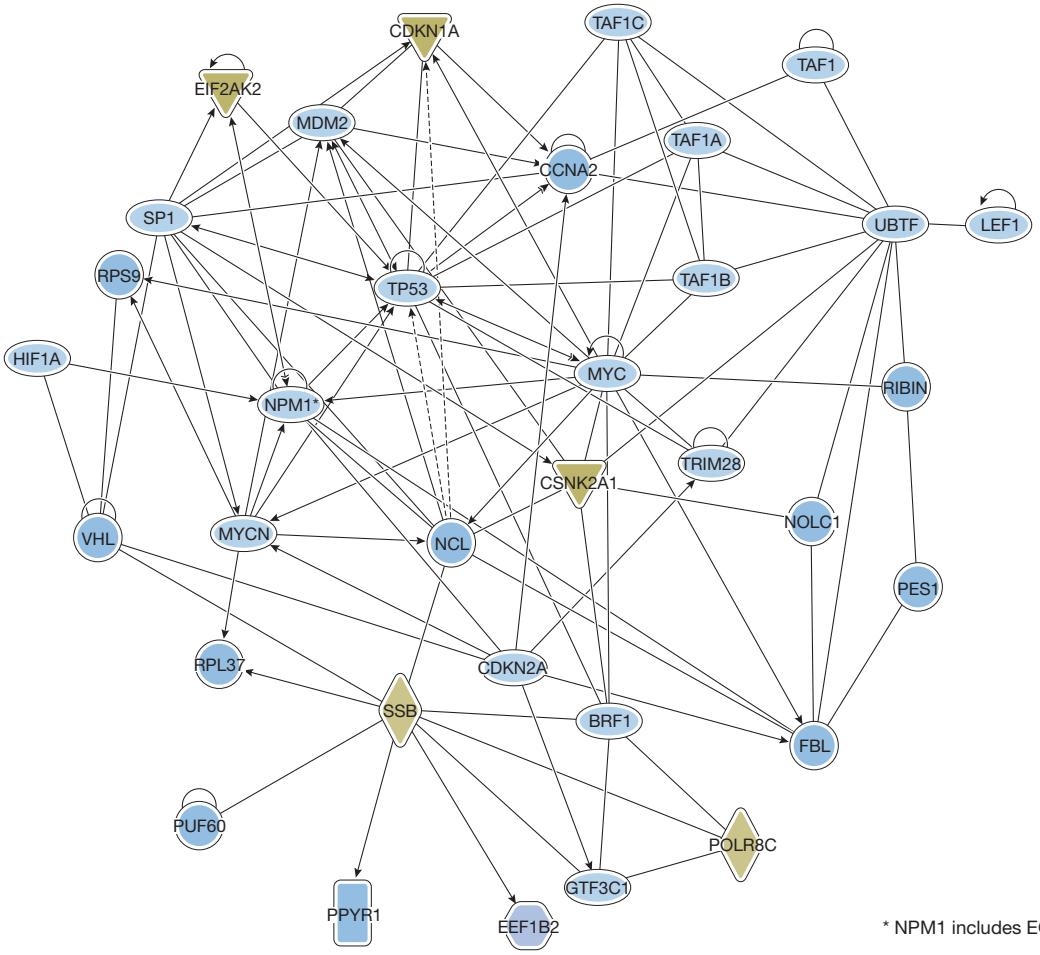

Fig 2 | Pathway analysis of direct interactions between selected nucleolar proteins, and other nuclear and cytoplasmic proteins. This interaction map was generated using Ingenuity Pathway Analysis software (Ingenuity Systems, Inc., Redwood City, CA, USA), and shows the complexity of the interactions—many of which are interlinked—and how perturbations to one protein might affect the function of another. Examples of proteins that can traffic to the nucleolus and that are discussed in the text, such as VHL, are also shown. The full names of all of the proteins that are shown on the map can be found in supplementary Table 1 online. FBL, fibrillarin; NCL, nucleolin; NPM1, nucleophosmin; VHL, von Hippel–Lindau tumour-suppressor protein.

the observation that many of the NoLSs for which binding partners are known interact with the same small subset of nucleolar proteins, thereby pointing to hub protein candidates, which include nucleolin and NPM. Whether such proteins are dynamic or static hubs is difficult to elucidate, and they might share characteristics of the two types. For example, both proteins can form multiple stable interactions throughout the cell cycle (Fig 2), which is a characteristic of static hubs; however, given the transitory nature of a subset of the nucleolar proteome—including ribosomal proteins, which are exported to the cytoplasm (Andersen *et al*, 2005)—they also show characteristics of a dynamic hub protein.

Nucleolin and NPM are predicted to contain disordered domains in an important part of the overall protein structure—corresponding to 55% and 47%, respectively (Fig 3)—which is a characteristic of hub proteins. Disordered proteins tend to have larger surface-to-volume ratios than ordered ones (Gunasekaran *et al*, 2003), and extensive surface areas are advantageous for binding to different ligands. Disordered proteins have been shown to interact with multiple protein or nucleic-acid partners, while maintaining an overall size that is two to three times smaller than ordered proteins (Gunasekaran *et al*, 2003); this characteristic is also advantageous for proteins that act as scaffolds for dense compartments such as the nucleolus, which would otherwise be considerably larger. Furthermore, disordered domains have been proposed to be involved in nucleolar protein–RNA interactions (Yiu *et al*, 2006). The known importance of nucleolin and NPM for the formation of nucleoli also supports their roles as hub proteins. As nucleoli breakdown and reform during M phase, the idea that such disruption could be controlled by affecting a small number of proteins that are crucial for maintaining the structural integrity of nucleoli has obvious advantages (Ma *et al*, 2007). The ablation of either nucleolin or NPM using RNA interference is sufficient to disrupt the nucleolar structure, although the exact nature of the disruption varies between the two proteins (Amin *et al*, 2008; Ma *et al*, 2007; Ugrinova *et al*, 2007). The depletion of NPM results in an accumulation of SUMOylated proteins within nucleoli (Yun

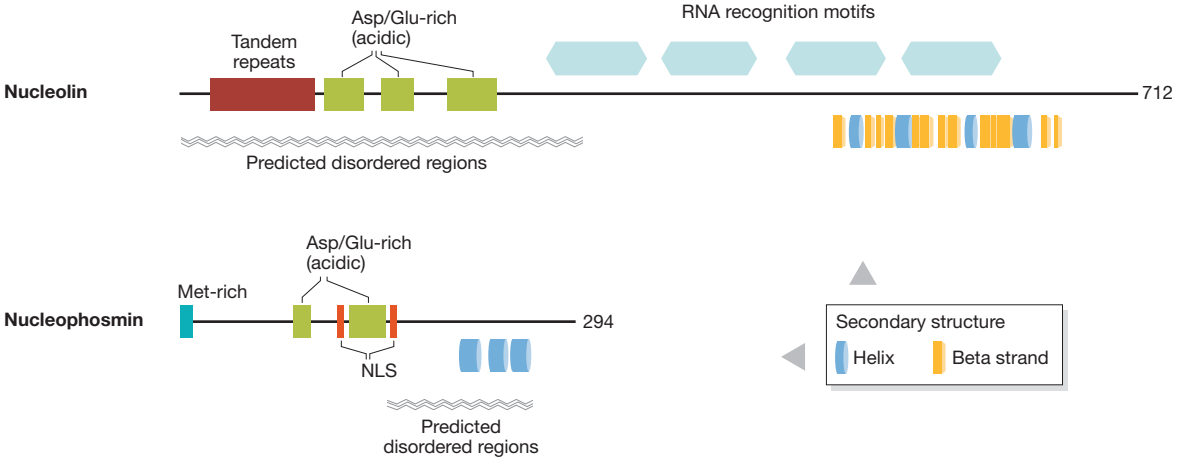

Fig 3 | Diagrammatic illustration of nucleolin and nucleophosmin. Various protein domains are highlighted, as well as the consensus results of a disorder analysis that was performed using several disorder predictors (see supplementary Fig 1 and supplementary Table 2 online for details of this analysis). NLS, nuclear localization signal.

et al, 2008), whereas the depletion of nucleolin results in the accumulation of cells in the G2 phase of the cell cycle, apoptosis, centrosome-control deficiencies and nuclear deformities, including multiple nuclei, micronuclei and large nuclei (Ugrinova *et al*, 2007). These findings illustrate the concept of the centrality–lethality rule, whereby the deletion of a hub protein is more likely to be lethal than the deletion of a non-hub protein (He & Zhang, 2006). Indeed, transgenic-knockout studies have shown that NPM is essential in the maintenance of genomic stability and embryonic development (Grisendi *et al*, 2005). Similarly, genetic mutations that cause the cytoplasmic localization of NPM and result in the stabilization of c-Myc are the most frequent alterations observed in acute myelogenous leukaemia (Bonetti *et al*, 2008). Conversely, overexpression of NPM can also contribute to the transforming ability of c-Myc (Li *et al*, 2008). In addition, changes in nucleolar structure can result from virus infection (Dove *et al*, 2006); nucleolin is dispersed by the UL24 protein of HSV-1, resulting in the alteration of nucleolar structure (Bertrand & Pearson, 2008; Lymberopoulos & Pearson, 2007).

Targeting proteins to and from the nucleolus

The localization of proteins to membrane-bound compartments such as the nucleus is mediated by dedicated targeting systems that recognize specific protein motifs and vary depending on protein size, hydrodynamic radius and concentration. Many proteins that traffic to and from the nucleus by the nuclear-pore complex contain one or more nuclear localization signals (NLSs) and, if necessary, also nuclear export signals (NESs). These motifs are well characterized and moderately conserved, and can therefore be predicted by suitable algorithms (Cokol *et al*, 2000; la Cour *et al*, 2004). However, the motifs that are involved in regulating nucleolar localization are not well defined and it is believed that nucleolar localization of a protein results from either direct or indirect interaction with one of the nucleolar building blocks—that is, with rDNA, its transcripts or protein components (Carmo-Fonseca *et al*, 2000). Therefore, rather than a targeting signal that acts as a recognition motif for the binding to import machinery, such signals would be responsible for high-affinity interactions with proteins or nucleic acids that reside within the nucleolus and thereby impart localization. Initial support for this hypothesis came from early studies of nucleolar proteins (Schmidt-Zachmann & Nigg, 1993); for example, although nucleolin was shown to contain a NLS, no defined NoLS could be identified, and it was therefore postulated that RNA-binding domains that were present within nucleolin were responsible for nucleolar accumulation. Similarly, other proteins that localize to the nucleolus might do so by binding directly to dsRNA, such as the NRF (Niedick *et al*, 2004).

Nucleolin has been shown to associate with NPM—which contains a NoLS—and therefore probably transports nucleolin to the nucleolus (Li *et al*, 1996). NPM can also associate with the ARF tumour-suppressor protein and retarget it to the nucleolus, where its function is inhibited (Korgaonkar *et al*, 2005). Another example of a protein that can localize to the nucleolus in the absence of a specific NoLS is PP1. In this case, nucleolar localization is driven by the NoLS-containing nucleolar protein NOM1 (Gunawardena *et al*, 2008). The association with NPM and other nucleolar proteins is also necessary for the trafficking and function of several viral proteins (Hiscox, 2007) such as the ORF3 protein of the plant umbravirus groundnut rosette virus, which associates with fibrillarin for the formation of viral ribonucleoprotein particles, the nucleolar translocation of which is essential for infection (Kim *et al*, 2007).

In some cases, the NoLSs are part of the NLSs, making their identification problematic and again pointing to the lack of consensus. FGF2—which regulates cell proliferation—contains two NLSs and the carboxy-terminal one also acts as a NoLS (Sheng *et al*, 2004). The ORF57 protein of HSV contains two NLSs, both of which are involved in nucleolar localization (Boyne & Whitehouse, 2006). Parafibromin—which is a tumour-suppressor protein—contains a bipartite NLS and three distinct NoLSs, all of which contribute to the efficiency of the nucleolar localization of this protein (Hahn & Marsh, 2007).

Table 1 | Nucleolar localization sequences

Protein	NoLS sequence	Position	Accession number	References
IBV N protein	WRRQARFK	71–78	AAA46214	Reed *et al*, 2006
ApLLP	MAKSIRSKHRRQMRMMKRE	1–19	ABY66901	Kim *et al*, 2003
HIV-1 TAT	RKKRRQRRRAHQ	48–61	AAC82591	Siomi *et al*, 1990
GGNNVα	RRRANNRRR	23–31	ACB21052	Guo *et al*, 2003
Angiogenin	IMRRRGL	53–59	AAA51678	Lixin *et al*, 2001
HSV type 1 γ(1)34.5	MARRRRHRGPRRPRPP	1–16	P08353	Cheng *et al*, 2002
HIV-1 REV	RRNRRRRWRERQRQI	38–52	CAA41586	Cochrane *et al*, 1990
FGF2	RSRKYTSWYVALKR	249–262	NP_001997	Sheng *et al*, 2004
MDM2	KKLKKRNK	466–473	Q00987	Lohrum *et al*, 2000
NIK	RKKRKKK	143–149	Q99558	Birbach *et al*, 2004
Nuclear VCP-like protein	KRKGKLKNKGSKRKK	112–126	NP_996671	Nagahama *et al*, 2004
p120	SKRLSSRARKRAAKRRLG	40–57	P46087	Valdez *et al*, 1994
HIC p40	GRCRRLANFGPRKRRRRRR	44–62	Q9P1T7	Thebault *et al*, 2000
MDV MEQ protein	RRRKRNRDARRRRRKQ	62–78	AAB48631	Liu *et al*, 1997
HVS ORF57	KRPR-RRPSRPFRKP (a bipartite NoLS; 25 residues separate the two halves in wild type, but it retains its functionality when they are joined)	91–94, 119–128	NP_040259	Boyne &Whitehouse, 2006
LIMK2	KKRTLRKNDRKKR	491–503	CAG30399	Goyal *et al*, 2006
PRRSV N protein	PGKKNKKKNPEKPHFP LATEDDVRHHFTPSER	41–72	AAG13733	Rowland *et al*, 1999; Rowland *et al*, 2003

ApLLP, *Aplysia* LAPS18-like protein; FGF2, fibroblast growth factor 2; GGNNVα, betanodavirus greasy grouper (*Epinephelus tauvina*) nervous necrosis virus protein α; HIC p40, human I-mfa domain-containing protein, p40; HIV-1 Rev, human immunodeficiency virus-1 regulator of virion protein; HIV-1 TAT, human immunodeficiency virus-1 transactivator of transcription protein; HVS, herpesvirus saimiri; HSV type 1 γ(1) 34.5, herpes simplex virus type 1 γ(1) 34.5 protein; IBV N, infectious bronchitis virus nucleocapsid; LIMK2, LIM kinases 2; MDM2, murine double minute 2 protein; MDV, Marek disease virus; MEQ, MDV Eco Q; NIK, nuclear factor-κB inducing kinase; NoLS, nucleolar localization signal; ORF57, open reading frame 57; PRRSV N, porcine reproductive and respiratory syndrome virus nucleocapsid; VCP, nuclear valosin-containing protein.

Several motifs have been identified that can be both necessary and sufficient—that is, they will direct an exogenous protein—to target a protein to the nucleolus; these are composed mostly of Arg or Lys residues. Such motifs can range in size from short sequences of seven or eight amino acids to approximately 30 residues (Table 1; Birbach *et al*, 2004; Reed *et al*, 2006). For example, studies of the NoLS of the human La protein and sequence alignment with other motifs indicated that the (R/K)(R/K)X(R/K) motif appeared once or as multiple copies in peptides that were targeted to the nucleolus (Horke *et al*, 2004); such motifs had been known since the discovery of NoLSs in HIV-1 and cellular proteins (Hatanaka, 1990; Dang & Lee, 1989). The conjugation of defined NoLSs to fluorescent proteins such as EGFP indicates that the motifs responsible for the function of a protein within the nucleolus can be distinct from those involved in nucleolar localization. For example, the nucleocapsid protein of the PRRSV—which binds to viral RNA—travels between the nucleolus and the cytoplasm (Rowland *et al*, 1999; You *et al*, 2008), and has separate motifs that are responsible for NoLS activity and fibrillarin binding, the association to which might cause defects in rRNA production (Rowland *et al*, 2003; Yoo *et al*, 2003). Presumably, nucleolar proteins that are above the size exclusion limit of the nuclear pore complex might also require a NLS for active transport through the nuclear envelope. This might explain why many NLSs and NoLSs contain shared motifs, as genetic stability and the availability of interfaces that are accessible for interaction with the trafficking machinery would drive the selection for proximal motifs.

NoLSs might be targets for other nucleolar proteins or RNAs. In this regard, NPM has been shown to interact with an artificial NoLS to direct exogenous proteins to specific compartments within the nucleolus (Lechertier *et al*, 2007). Indeed, the fusion of the same artificial NoLS with another nucleolar protein, fibrillarin—which contains its own NoLS—showed that it could be retargeted from the DFC to the GC by conferring on fibrillarin high affinity for NPM (Lechertier *et al*, 2007). This suggests that NoLSs might have a hierarchal dominance in terms of directing proteins to the nucleolus and its subcompartments. Swapping NoLSs between viral proteins that have different nucleolar localization and trafficking rates has certainly illustrated that the NoLS can determine both of these properties, although different NoLS sequences might confer the same biological activity (Emmott *et al*, 2008). For example, abolishing the NoLS of the ORF57 protein of

Sidebar A | In need of answers

(i) The generation of a dynamic interactome map of the nucleolus would aid the prediction of loss and/or gain of function through the sequestration and release of nucleolar proteins as a result of disease.

(ii) How can nucleolar localization signals (NoLSs) be identified using bioinformatics-based approaches? Several studies are now beginning to address this problem (Boden & Teasdale, 2008).

(iii) Complete structural information on native nucleolar proteins with and without appropriate post-translational modifications would help to determine how they might signal or control the activity of the NoLS.

HVS prevented the trafficking of intronless HVS RNA from the nucleolus to the cytoplasm, yet this activity was rescued by the addition of the NoLS of the HIV-1 Rev protein (Boyne & Whitehouse, 2006).

Several factors might be responsible for determining the activity of NoLSs. Similar to the positioning of NESs, NoLSs might be situated on the exterior of the parent protein, making them available for interaction with other molecules that are thereby also targeted to the nucleolus (Horke *et al*, 2004). The activity of NoLSs might also be regulated by post-translational modifications; a notable example of this is found in the NoLS and NES of the HSV-1 US11 protein, which comprises a single shared proline-rich motif the activity of which—to mediate nucleolar localization or nuclear export—is regulated by phosphorylation (Catez *et al*, 2002). This finding also emphasizes the fact that Arg and Lys residues are not the sole functional residues of NoLSs, as is the case with NPM, for which Trp residues were reported to be important (Nishimura *et al*, 2002). The subcellular localization of NPM is also controlled by SUMOylation, which occurs in close proximity to the NoLS motif (Liu *et al*, 2007) and can affect the role of NPM in 28S rRNA maturation (Haindl *et al*, 2008).

Conclusion

The use of proteomics and fluorescent live-cell imaging has rapidly expanded the repertoire of known nucleolar proteins and our understanding of their functions. These proteins might be resident in the nucleolus throughout interphase or their localization might be dependent on the metabolic state of the cell. The localization of proteins to the nucleolus is undoubtedly dynamic, and requires suitable signalling and trafficking cascades. The challenge in understanding nucleolar trafficking is separating these cascades in order to build a complex model that illustrates nucleolar dynamics, from the beginning of nucleolar assembly at the end of mitosis to the subsequent nucleolar disassembly at the end of cell division. The stoichiometry of proteins in the nucleolus is crucial for its successful functioning, as the release or sequestration of proteins to the nucleolus has profound biological consequences. The study of the nucleolar proteome has revealed families of proteins that localize in the nucleolus, thereby increasing the chances of discovering potential classes of NoLSs and their interacting partners. The nucleolus might be built around hub proteins that allow the binding of multiple protein partners, which could be orchestrated in a position-dependent and time-dependent manner, in that both localization within the nucleolus and the trafficking rate could be determined by the NoLS. These NoLSs might be recognized by the hub nucleolar proteins, which are centred around the rDNA (see sidebar A). There are clearly various classes of NoLSs, and their identification, characterization and classification will undoubtedly increase our understanding of nucleolar targeting and formation.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org)

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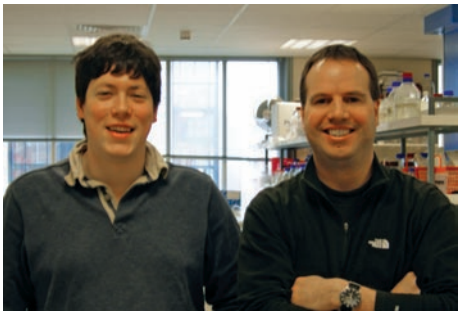

Edward Emmott (left) & Julian A. Hiscox

